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Quinolone Resistance-Determining Region in the DNA Gyrase *gyrB* Gene of *Escherichia coli*

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Thirteen spontaneous quinolone-resistant *gyrB* mutants of *Escherichia coli* KL16, including two that were examined previously, were divided into two types according to their quinolone resistance patterns. Type 1 mutants were resistant to all the quinolones tested, while type 2 mutants were resistant to acidic quinolones and were hypersusceptible to amphoteric quinolones. Nucleotide sequence analysis disclosed that all nine type 1 mutants had a point mutation from aspartic acid to asparagine at amino acid 426 and that all four type 2 mutants had a point mutation from lysine to glutamic acid at amino acid 447.

Quinolones are a group of antibacterial agents whose target is DNA gyrase (EC 5.99.1.3), an enzyme that catalyzes topological changes of DNA (4). The DNA gyrase of *Escherichia coli* consists of two A and two B subunits, which are the products of the *gyrA* (48 min) and *gyrB* (83 min) genes, respectively (3, 7, 11, 21, 29). Mutations in the *gyrA* gene are as frequent as those in the *gyrB* gene in spontaneous quinolone-resistant mutants of *E. coli* KL16, although the majority of quinolone-resistant clinical *E. coli* isolates have *gyrA* mutations (23). In the *gyrA* gene, quinolone resistance is caused by a point mutation within the relatively narrow region of amino acids 67 to 106, which is called the quinolone resistance-determining region (34, 35). In the *gyrB* gene, two quinolone resistance-determining sites (amino acids 426 and 447) have been found (32, 33). To obtain more information on the region responsible for quinolone resistance in the *gyrB* gene, 11 additional quinolone-resistant *gyrB* mutants of *E. coli* KL16 were analyzed.

MATERIALS AND METHODS

Strains. Quinolone-resistant mutants of *E. coli* KL16 were isolated by plating the organism on LB agar (18) containing nalidixic acid or enoxacin at four times the MIC, and *gyrB* mutants were identified by transformation with the wild-type *gyrB* gene as described previously (23).

Reagents, plasmids, and phages. Nalidixic acid (14), oxolinic acid (10), cinoxacin (30), piromidic acid (19), flumequine (24), pipemidic acid (15), norfloxacin (12), enoxacin (16), ofloxacin (6), ciprofloxacin (5), tosufloxacin (1), and sparfloxacin (formerly AT-4140) (20, 22) were synthesized in our laboratories. Plasmid vector pBR322, restriction endonucleases, T4 DNA ligase, a sequencing kit, and the phage M13mp18 and M13mp19 vectors were purchased from Takara Shuzo Co., Ltd.; [α-^{32}P]dCTP (>400 Ci/mmol) was purchased from Amersham International; and other reagents were purchased from Nacalai Tesque, Inc.

Preparation of DNA. Chromosomal DNA was prepared by the method of Cosloy and Oishi (2). Small-scale plasmid DNA isolation was carried out by the rapid boiling method described by Holmes and Quigley (8), and large-scale plasmid isolation was carried out by the method of Wilkie et al. (31).

Transformation. Transformation was performed by the $CaCl_2$ method, and transformants were selected on LB agar containing ampicillin at 25 μg/ml.

Cloning and sequencing of the *E. coli gyrB* genes. *Hin*dIII DNA fragments of about 13 kb in size containing the *gyrB* gene were cloned from quinolone-resistant *gyrB* mutants of *E. coli* KL16 as described previously (33). Nucleotide sequences were determined by the dideoxy-chain termination method (17) by using phage M13mp18 and M13mp19 vectors.

RESULTS AND DISCUSSION

The levels of resistance or hypersusceptibility (the increase or decrease in MIC compared with that for *E. coli* KL16) to various quinolones of 13 quinolone-resistant *gyrB* mutants of *E. coli* KL16 are given in Table 1. The MICs of some quinolones for N-24 and N-31 were not identical to those reported previously (23, 32) but were within experimental fluctuations. All the mutants could be divided into two types with respect to their quinolone resistance. Type 1 mutants were resistant to all the quinolones tested, while type 2 mutants were resistant to acidic quinolones, such as nalidixic acid, oxolinic acid, cinoxacin, piromidic acid, and flumequine but were hypersusceptible to amphoteric quinolones, such as pipemidic acid, norfloxacin, enoxacin, ofloxacin, ciprofloxacin, tosufloxacin, and sparfloxacin. Nine mutants (N-24, N-102, N-103, N-107, N-108, N-111, N-114, EN-2, and EN-5) belonged to type 1, and four mutants (N-31, N-109, N-115, and N-120) belonged to type 2. All 13 mutants were considered to have a mutation responsible for the quinolone resistance on the 1.2-kb *Cla*I fragment of the *gyrB* gene (33), because replacement of the *Cla*I fragment by the corresponding fragment from the wild-type *gyrB* gene resulted in the complete loss of quinolone resistance. Sequencing of the entire regions of the *Cla*I fragments of all the mutants revealed that all the type 1 mutants, including N-24 (analyzed previously [33]), had a G-to-A transition at the first base of codon 426, resulting in an amino acid change from aspartic acid to asparagine, and that all the type 2 mutants, including N-31 (analyzed previously [33]), had an A-to-G transition at the first base of codon 447, resulting in an amino acid change from lysine to glutamic acid. These unexpected results suggest that only such mutations cause quinolone

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TABLE 1. Quinolone resistance of *E. coli* KL16 *gyrB* mutants

Strain[a]	MIC (μg/ml)												Mutation
	Acidic quinolones[b]					Amphoteric quinolones[c]							
	NA	OA	CINO	PA	FLQ	PPA	NFLX	ENX	OFLX	CPFX	TFLX	SPFX	
Type 1 mutants													
N-24	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-102	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-103	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-107	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-108	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-111	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
N-114	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
EN-2	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
EN-5	50	1.56	12.5	100	1.56	12.5	0.39	0.78	0.39	0.1	0.05	0.05	Asp-426 → Asn
Type 2 mutants													
N-31	50	1.56	12.5	50	1.56	0.39	0.0125	0.025	0.0125	0.0031	0.0031	0.0031	Lys-447 → Glu
N-109	50	1.56	12.5	50	1.56	0.39	0.0125	0.025	0.0125	0.0031	0.0031	0.0031	Lys-447 → Glu
N-115	50	1.56	12.5	50	1.56	0.39	0.0125	0.025	0.0125	0.0031	0.0031	0.0031	Lys-447 → Glu
N-120	50	1.56	12.5	50	1.56	0.39	0.0125	0.025	0.0125	0.0031	0.0031	0.0031	Lys-447 → Glu
KL16	3.13	0.39	3.13	6.25	0.39	1.56	0.05	0.1	0.05	0.0125	0.0125	0.0125	Wild type

[a] Strains EN-2 and EN-5 were selected with enoxacin, and the other strains (N-24 to N-120) were selected with nalidixic acid.
[b] NA, nalidixic acid; OA, oxolinic acid; CINO, cinoxacin; PA, piromidic acid; FLQ, flumequine.
[c] PPA, pipemidic acid; NFLX, norfloxacin; ENX, enoxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; TFLX, tosufloxacin; SPFX, sparfloxacin.

resistance or have a selective advantage under the selective conditions used for *E. coli* KL16.

As shown in Fig. 1, there are three hydrophilic valleys, with bottoms at amino acids 425, 437, and 448, in the hydrophobicity profile of the quinolone resistance-determining region of the GyrB protein, according to the algorithm of Hopp and Woods (9). It is likely that such hydrophilic areas constitute the surface of the GyrB protein in an aqueous solution. In addition, it was predicted, by the method of Rose (25), that the polypeptide chain for the quinolone resistance-determining region had turns around amino acids 425–426 and 448. Therefore, both the mutation sites, Asp-426 and Lys-447, would be situated at or near the turn loci on the GyrB protein surface. If the mutation sites were close to each other topologically, the minus charge of Asp-426 and the plus charge of Lys-447 might interact with each other to yield neutral circumstances, for which hydrophobic groups, e.g., the methyl group of nalidixic acid, the methylenedioxy groups of oxolinic acid and cinoxacin, and the pyrrolidinyl group of piromidic acid, have high affinity (Fig. 2). The minus charge of Asp-426 could interact with the plus charge of piperazinyl and aminopyrrolidinyl groups at position 7 of amphoteric quinolones. In the type 1 mutant GyrB protein in which Asp-426 was replaced by Asn, the minus charge at amino acid 426 disappeared and the local conformation of the quinolone resistance-determining region would probably change because the hydrophobicity profile of the region was

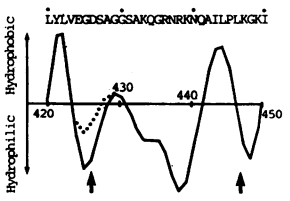

FIG. 1. Hydrophobicity profile of the quinolone resistance-determining region of the GyrB polypeptides of *E. coli* KL16, N-24, and N-31 by the algorithm of Hopp and Woods (9). The amino acid sequence of this region of the wild-type GyrB protein is given above the profile. The solid line is for all three strains except for the region between amino acids 424 and 428, where the solid line is for both the wild-type strain KL16 and the type 2 (Lys-447 to Glu) mutant N-31 and the dotted line is for the type 1 (Asp-426 to Asn) mutant N-24. The locations of the mutations are indicated by arrows.

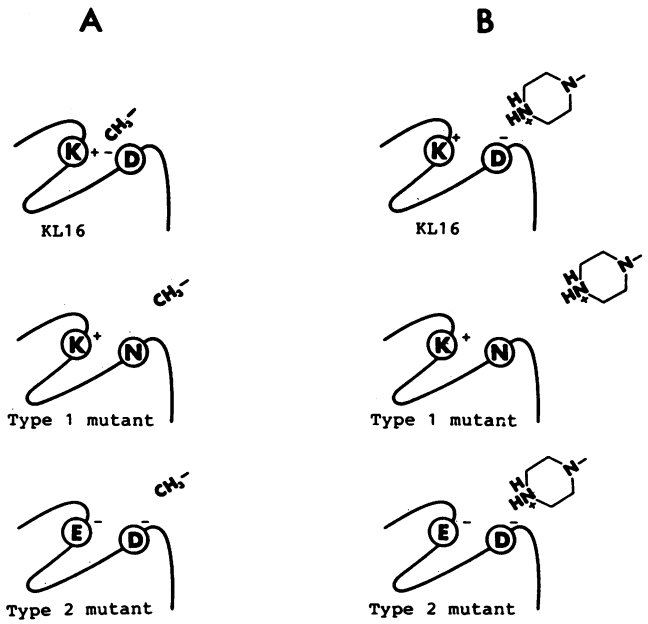

FIG. 2. Hypothetical interaction of GyrB protein with the groups of quinolones at position 7. (A) Interaction with the methyl group of the nalidixic acid at position 7. (B) Interaction with the piperazinyl group of new quinolones. D, Asp-426; N, Asn-426; K, Lys-447; E, Glu-447.

significantly different from that for the wild-type GyrB protein (Fig. 1). Such a change would make it difficult for a hydrophobic or positively charged group of quinolones to associate with the region (Fig. 2). In the type 2 mutant GyrB protein in which Lys-447 was replaced by Glu, only a change in charge from plus to minus occurred at amino acid 447, without a change in the hydrophobicity profile (Fig. 1). Such a change makes the region a negatively charged one, with which a positively charged group but not a hydrophobic one is likely to interact (Fig. 2), consistent with the fact that the type 2 mutants are resistant to acidic quinolones with a hydrophobic group at position 7 of the skeletal rings but are hypersusceptible to amphoteric quinolones with a positively charged group at the corresponding position. However, this interpretation is hypothetical at present, and we cannot rule out the other possibilities that mutations may cause allosteric conformational changes and the quinolone resistance-determining region may not really interact with quinolones.

Shen et al. (27) have reported that a new quinolone-binding site appears in a gyrase-DNA complex after complex formation and that the inhibition of gyrase by quinolones occurs as a result of quinolone binding to the site. We found that quinolone resistance was caused by a mutation within the region of amino acids 67 to 106, especially in the vicinity of amino acid 83, of the GyrA protein, which is close to Tyr-122, the binding site of transiently cleaved DNA, and proposed the idea that the region might be involved in or near the quinolone-binding site (34). The present study disclosed that quinolone resistance is also caused by a mutation at either amino acid 426 or 447 of the GyrB protein and that a group at position 7 of quinolones might interact with the region that includes the mutation sites. Therefore, we postulate that the quinolone-binding site may be a small pocket bounded by surfaces involved with or near the quinolone resistance-determining regions of both the GyrA and GyrB proteins, which we call the quinolone pocket. The results of neutron and light-scattering studies support the existence of channels or cavities in gyrase whose shape changes very little upon the addition of DNA (13).

Shen et al. (26–28) proposed a cooperative quinolone-DNA binding model as the molecular mechanism for the ternary complex formation between a quinolone, DNA, and gyrase, in which the interaction of a quinolone with single-stranded DNA and the self-association of the quinolone are assumed. However, our finding that quinolone resistance depends on local conformational and/or ionic changes of either the GyrA or GyrB protein suggests that the quinolone-gyrase interaction in the quinolone pocket is responsible for the gyrase sensitivity to quinolones, and that the quinolone–single-stranded DNA interaction and the self-association of quinolones, if any, may have little relevance to it. Recently, Willmott et al. (31a) reported that the gyrase reconstituted from the mutant GyrA (Ser-83 to Ala) protein and the wild-type GyrB protein is resistant to ciprofloxacin in the supercoiling, decatenation, relaxation, and cleavage reactions and that its complex with DNA binds less norfloxacin than the complex formed with wild-type gyrase and DNA does. This indicates the relationship between a quinolone resistance mutation and quinolone binding to a gyrase-DNA complex. Further studies on quinolone binding with gyrases reconstituted from wild-type and various mutant GyrA and GyrB proteins will clarify the characteristics of the quinolone pocket.

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